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Blood lymphocyte subpopulations in breast cancer patients following radiotherapy

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SUMMARY

Both T and non-T lymphocytes decreased immediately following radiotherapy in breast cancer patients. The relative depletion of non-T lymphocytes, however, was more marked than that of T cells. 3 years later the number and the proportion of non-T lymphocytes was higher than immediately after radiotherapy, while T lymphocytes were still depressed. The proportion of cells with membrane-associated Ig was higher in patients 3 years following radiotherapy than in non-treated patients and healthy controls. There was no difference in the proportion of T and non-T lymphocytes between patients with and without metastases, respectively.

INTRODUCTION

Local radiotherapy applied to different parts of the human body induces peripheral lymphopenia (Goswitz, Andrews & Kniseley, 1963; Millard, 1965; Meyer, 1970; Ilbery, Rickinson & Thrum, 1971; Thomas *et al.*, 1971; Stjernswärd *et al.*, 1972; McCredie, Inch & Sutherland, 1972; Glas & Wasserman, 1974; Blomgren *et al.*, 1974; Heier *et al.*, 1975). Conflicting data concerning the relative proportions of T and B lymphocytes in blood after radiotherapy have, however, been presented. Stjernswärd *et al.* (1972) and Raben *et al.* (1976) demonstrated a relative T-cell depletion, whereas Anderson *et al.* (1975) and Campbell *et al.* (1976) found similar rates of reduction in T and B cells. In our earlier studies (Blomgren, Wasserman & Littbrand, 1974; Blomgren *et al.*, 1976) we have observed an increased proportion of T cells and decreased proportion of non-T cells immediately after irradiation therapy in patients with cancer of the breast, prostate gland or urinary bladder. Also Heier *et al.* (1975) reported similar findings in seminoma patients. Thus it was considered of interest to further investigate lymphocyte subpopulations in irradiated patients, using an additional marker for the identification of non-T lymphocytes. For this reason we have re-examined patients who 3 years ago were investigated immediately after the completion of radiotherapy. The aim of the present investigation was to study the repopulation of T and non-T cells during recovery from the lymphopenia induced by radiotherapy.

In previous studies we have demonstrated, in patients with breast cancer, that the *in vitro* stimulation of lymphocytes with PPD is impaired at the time of discovery of metastases (Glas *et al.*, 1976). A shift in the lymphocyte subpopulations could be the reason for this decreased responsiveness of lymphocytes. Therefore proportions of lymphocyte subpopulations in patients with metastases were examined and compared with those in controls.

MATERIALS AND METHODS

Tests used. T lymphocytes were identified by means of E-rosette tests and non-T lymphocytes by EAC-rosette tests. Moreover, the determination of membrane-associated Ig was used for evaluation of non-T lymphocytes. As this surface

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marker was not examined initially, the values obtained 3 years after irradiation were compared to those of untreated breast cancer patients and healthy subjects. Receptor structures for activated C3 (EAC) are present on B lymphocytes but also on the surface of a third lymphocyte population which comprises of so-called K cells (Perlmann & Perlmann, 1970). Furthermore, it is possible that some B cells do not possess C3 receptors. B cells are usually identified by the presence of surface Ig. Such surface Ig, however, can either be truly incorporated into the cell membrane as in B cells, or be passively adsorbed to Fc membrane receptors which are to be found on cells belonging to the above-mentioned third lymphocyte subpopulation (Lobo, Westervelt & Horwitz, 1975). The method used in this investigation does not permit differentiation between these two and consequently does not allow discrimination between B cells and other non-T cells, which will both be referred to as non-T cells.

Surface markers. T cells were determined by E-rosette formation according to Jondal, Holm & Wigzell (1972) and non-T cells were identified by EAC-rosette formation (Jondal *et al.*, 1972) and by the presence of membrane-associated immunoglobulin (Ig cells) detected by indirect immunofluorescence (Fröland *et al.*, 1972). Polyspecific rabbit anti-Ig (Cappel Lab., Pennsylvania, U.S.A.) and fluorescein-isothiocyanate (FITC) conjugated sheep anti-rabbit Ig (National Bacteriological Laboratory, Stockholm) were used. A Leitz Orthoplan microscope with an Osram HBO 100 mercury lamp and vertical illuminator was employed. Filters were 4 mm BG 38+S470+2xKP 490 and secondary filters 515+510. Using oil-immersion objective 54/95 the proportion of Ig cells was determined by alternate illumination with conventional light and light specific for FITC fluorescence. At least 200 cells were counted.

Purification of lymphocytes. Heparinized blood was centrifuged over a Ficoll–Isopaque (FIP) gradient (Böyum, 1968). Phagocytic cells were removed by iron powder and a magnet (Lundgren, Zukoski & Möller 1968). The lymphocytes were then once again centrifuged on FIP to remove all remaining erythrocytes and granulocytes. The resulting preparation was extremely pure (99–100% small lymphocytes). The lymphocyte recovery efficiency was about 30% in breast cancer patients (unpublished results). However, no selection of any lymphocyte subpopulation was detectable (Petrini, to be published).

In patients investigated 3 years ago another technique was used at that time. Defibrinated blood was treated by gelatine sedimentation (Coulson & Chalmers, 1964), followed by nylon-wool purification to remove adherent cells (Perlmann & Perlmann, 1970) and subsequent FIP centrifugation. When these patients were now re-investigated this former technique was employed in parallel with the above-described method with double FIP centrifugation. Using both these techniques no significant differences concerning the lymphocyte subsets were detectable (Petrini, to be published).

Radiotherapy. The post-operative radiotherapy was directed to the axilla, infra- and supra-clavicular regions, both retrosternal regions and the chest wall: the pre-operative radiotherapy was directed to the same regions and to the mammary gland. The pre-operative as well as the post-operative treatment of the chest wall were planned individually. Radiotherapy was given by ^{60}Co gamma-irradiation or by high-energy electrons. The tumour dose was 4,500 rad over a period of 6 weeks. Surgery was performed 6 weeks after the completion of radiotherapy or 5–6 weeks prior to irradiation.

Statistical method. Probability values were determined using Student's *t*-test. (Lindgren, 1969; Cramér, 1946).

Patients. In total eighty women with primary carcinoma of the breast were investigated. For data concerning diagnostic procedure and treatment see Franzén & Zajicek (1968), Glas & Wasserman (1974) and de Schryver (1975).

In twenty-nine women treated by pre- or post-operative radiotherapy, or surgery only, the lymphocyte subpopulations before and immediately after treatment were determined. Ten of the patients were treated by surgery only and served as controls. They were tested with the same reagents and time intervals as the radiotherapy-treated patients. The test system for E and EAC cells used by us has been investigated earlier without any significant fluctuations between different test occasions being noted (Blomgren *et al.*, 1974).

In ten other women, irradiated and examined 3 years previously and now free of metastases, T and non-T cells were examined and the values compared to those found before and immediately after radiotherapy. For trivial reasons differential counts were not obtained in all these patients. The proportion of Ig cells, which was not examined 3 years earlier in these patients, was compared to that of twenty newly diagnosed breast cancer patients and nineteen healthy age-matched staff members.

The relation between the presence of metastases and the distribution of lymphocyte subpopulations was studied in forty-one patients. Fourteen of these patients had metastases and were all examined within 1 year after the discovery of these. The duration between the time of diagnosis of primary breast cancer and the present investigation was from 6 months to 7 years, in most cases 3 years. Twenty-seven controls were breast cancer patients free of metastases with approximately the same interval between the time of cancer diagnosis and lymphocyte examination. Some of the patients received radiotherapy pre- or post-operatively 1–4 years before the present examination. The above-mentioned ten patients examined 3 years after radiotherapy were included in this control group. None of the patients received cytostatic drugs or corticosteroids at the time of investigation but two patients with metastases received 100 mg of an androgenic steroid every 14 days (Primobolan®, Depot, Schering AG, Germany) and one an antioestrogenic drug (Tamoxifen).

RESULTS

Lymphocytes before and immediately after radiotherapy

The patients treated by pre- or post-operative radiotherapy were pooled. The pre-treatment and

post-treatment values for lymphocytes and their subpopulations are given in Table 1. The total lymphocyte counts decreased significantly after radiotherapy ($P<0.001$) as well as the number of E, EAC and Ig cells. However, when determining the relative proportions of lymphocyte subpopulations before and immediately after radiotherapy, respectively, it was demonstrated that the proportion of E cells increased significantly whereas the proportion of EAC and Ig cells decreased significantly (Table 1). In patients treated by surgery only, no significant alteration in total number or proportion of lymphocyte subpopulations was observed following the operation (Table 1).

TABLE 1. Effect of radiotherapy on lymphocytes and lymphocyte subpopulations in breast cancer patients

	Radiotherapy		Statistical difference	Surgery only		Statistical difference
	Before	After		Before	After	
Lymphocytes/μl	2072±255	649±151	$P<0.001$	2204±806	2017±959	n.s.
E cells (%)	55±5	60±5	$P<0.05$	58±8	61±3	n.s.
EAC cells (%)	26±4	21±3	$P<0.01$	25±3	23±7	n.s.
Ig cells (%)	21±4	13±3	$P<0.01$	19±5	21±8	n.s.
E cells/μl	1113±148	379±85	$P<0.001$	1325±434	1120±481	n.s.
EAC cells/μl	552±108	143±51	$P<0.001$	559±245	487±313	n.s.
Ig cells/μl	442±107	91±39	$P<0.001$	458±317	497±415	n.s.

Means and 95% confidence limits. n.s. = Not significant.

Relative depletion of E and EAC cells immediately after radiotherapy

In radiotherapy-treated patients the ratios between the cell numbers after and before irradiation were calculated for E and EAC cells respectively (Table 2). The depletion of EAC cells was significantly more marked than that of E cells ($P<0.01$).

TABLE 2. Relative depletion of E and EAC cells per μl immediately after radiotherapy. Means and 95% confidence intervals of ratios after/before treatment

E after/E before	EAC after/EAC before	Statistical difference
0·39±0·06	0·25±0·08	$P<0.01$

Lymphocytes before, immediately after and 3 years after radiotherapy

3 years after radiotherapy the total number of lymphocytes was significantly higher than immediately after treatment but had not reached pre-treatment values (Table 3). These results are in line with earlier observations (Baral *et al.*, 1977). The numbers of E and EAC cells per μl of blood were higher 3 years after than immediately after irradiation, but the increase was significant only for EAC cells (Table 3). The proportions of EAC cells increased significantly 3 years after the treatment when compared to values obtained shortly after radiotherapy. The proportion of E cells, on the other hand, did not change significantly (Table 3). When comparing the lymphocyte numbers per μl and the proportions of E and EAC cells obtained before radiotherapy and 3 years after, the only significant difference was the depression of the number of E cells per μl (Table 3). The kinetics of the depletion and repopulation of lymphocytes and lymphocyte subpopulations are schematically presented in Fig. 1.

In patients treated with radiotherapy 3 years earlier the proportion of Ig cells was significantly higher than in untreated breast cancer patients and healthy subjects (Table 4). The proportion of Ig cells was also higher in untreated patients than in healthy controls (Table 4).

TABLE 3. Lymphocytes and lymphocyte subpopulations in breast cancer patients treated with radiotherapy (Rx)

	Means and 95% confidence intervals						Statistical differences		
	Before Rx (1)		Immediately after Rx (2)		3 yr after Rx (3)		(1) and (2)	(1) and (3)	(2) and (3)
Lymphocytes/μl	8*	2165±431	8	873±273	9	1606±349	$P<0{\cdot}01$	n.s.	$P<0{\cdot}05$
E cells (%)	10	52±7	10	60±4	10	53±6	$P<0{\cdot}05$	n.s.	n.s.
EAC cells (%)	10	21±5	10	9±4	10	28±7	$P<0{\cdot}001$	n.s.	$P<0{\cdot}001$
E cells/μl	8	1147±258	8	548±201	9	844±164	$P<0{\cdot}001$	$P<0{\cdot}01$	n.s.
EAC cells/μl	8	460±260	8	73±40	9	447±200	$P<0{\cdot}05$	n.s.	$P<0{\cdot}05$

n.s. = Not significant.

* Number of cases.

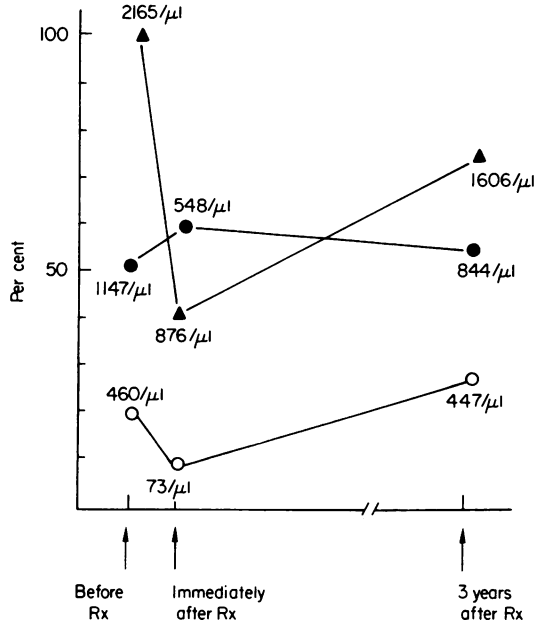

FIG. 1. Lymphocytes and proportions of subpopulations before, immediately after and 3 years after radiotherapy (Rx). Mean values of ten patients. (▲) Lymphocytes; (●) E cells; (○) EAC cells.

TABLE 4. Percentage of Ig cells in twenty untreated breast cancer patients, ten patients 3 years after radiotherapy (Rx) and nineteen healthy controls, respectively. Means and 95% confidence intervals

Untreated by Rx (1)	3 years after Rx (2)	Healthy controls (3)	Statistical differences		
			(1) and (2)	(1) and (3)	(2) and (3)
20±4	32±9	13±2	$P<0{\cdot}05$	$P<0{\cdot}01$	$P<0{\cdot}001$

TABLE 5. Lymphocytes and lymphocyte subpopulations in breast cancer patients with and without metastases. Means and 95% confidence intervals. The differences between the values of patients with and without metastases are not significant

	No radiotherapy		1–4 years after radiotherapy	
	With metastases ($n=7$)	Free of metastases ($n=11$)	With metastases ($n=7$)	Free of metastases ($n=16$)
Lymphocytes/μl	1731±765	1805±454	1317±616	1709±258
E cells (%)	56±6	60±4	56±6	52±4
EAC cells (%)	28±6	33±9	29±7	31±6
Ig cells (%)	18±11	25±10	29±7	34±6

Patients with metastases

There was no significant difference between the proportions of E cells, EAC cells and Ig cells in breast cancer patients with and without metastases examined at the same time after the primary treatment. This applied both to patients treated and not treated by adjuvant radiotherapy (Table 5).

DISCUSSION

The present results confirm our earlier findings concerning the more extensive depletion of the non-T cells than of the T cells during lymphopenia shortly after irradiation (Blomgren *et al.*, 1974, 1976). The results obtained with surface Ig as an additional marker of non-T cells were in line with those demonstrated with EAC-rosette tests (Blomgren *et al.*, 1974, 1976) (Tables 1 and 5). The conflicting evidence on this subject presented by Stjernswärd *et al.* (1972) and Raben *et al.* (1976) may be explained by their use of not completely purified lymphocyte preparations. The separation technique used by these investigators did not include removal of phagocytic cells. This step is rather important since it has recently been shown that the frequency of monocytes increase in breast cancer patients after radiotherapy (Lundell, 1974). Monocytes also form EAC rosettes which cannot easily be distinguished from rosettes formed by lymphocytes. If only Ficoll–Isopaque is used for separation of the lymphocytes, as it has been done in the above-cited studies, the interpretation of the test will be difficult, since the monocytes in the cell preparation will be markedly increased after radiotherapy. Our results concerning the immediate effect of radiotherapy on the frequency of lymphocyte subpopulations have recently been confirmed by Heier *et al.* (1975) in patients with seminoma. The findings of a lower number of T cells per μl of blood 3 years after irradiation as compared to before radiotherapy (Table 3) indicates that a part or a subpopulation of T cells has been eliminated. The low numbers and proportions of EAC cells found immediately after radiotherapy, on the other hand, were restored 3 years later to values not significantly different from those present before irradiation (Table 3). This is in agreement with other observations according to which low T- and high or normal B-cell proportions were found 1–2 years after radiotherapy (Stjernswärd *et al.*, 1972; Heier *et al.*, 1975; Anderson *et al.*, 1975; Campbell *et al.*, 1976). One explanation to these findings might be that T cells have a slower turnover than certain non-T cells (Trepel, 1975) and thus can be expected to repopulate less rapidly and possibly less completely than these.

The proportion of cells with membrane-associated Ig was high 3 years following radiotherapy as compared to untreated patients and healthy controls (Table 4). It is possible that also these data reflect a relatively rapid repopulation of non-T cells after irradiation although probably different subpopulations are indicated with the Ig and EAC techniques (Chiao *et al.*, 1975).

The demonstrated differences in the kinetics of depletion and repopulation between different lymphocyte subpopulations confirm some reports in the literature and are at variance with others. It should therefore be mentioned that the present experiments were performed with very pure lymphocyte

preparations which decreases the possibility of experimental errors. It is of interest in this context that the recovery of PPD response *in vitro* after radiotherapy progresses more rapidly than than the repopulation of the total lymphocyte population (Baral *et al.*, 1977). This recovery of the PPD reactivity of the cells could be due to the restitution of 'amplifier non-T lymphocytes' (Blomgren, 1975)—cells which might belong to the rapidly repopulated lymphocytes described in this report.

Untreated breast cancer patients at the time of diagnosis had a higher proportion of Ig cells than healthy controls (Table 4). There was no such difference as far as E and EAC cells are concerned (data not published). It is possible that this indicates that patients with breast cancer have an increased proportion of B cells. More extended studies with specific B-cell markers are, however, necessary before such a conclusion can be drawn. Babušiková *et al.* (1975) found lower proportion of T cells and higher proportion of B cells in cancer patients with distant metastases. This could not be verified in our investigation, when lymphocyte subpopulations were compared in patients with and without metastases (Table 5). The same results were obtained in groups treated or non-treated with radiotherapy (Table 5). However, the number of patients was limited, as most patients with metastases were on drug treatment and thus not suitable for lymphocyte studies.

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